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A Synthetic Camel Anti-Lysozyme Peptide Antibody (Peptibody) with Flexible Loop Structure Identified by High-Resolution Affinity Mass Spectrometry

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Abstract: We describe the synthesis and characterisation of the fully functional molecular recognition structure of a 26-amino acid residue peptide antibody, referred to as *peptibody*, designed from a monoclonal single-domain antibody fragment derived from a camel heavy-chain antibody. The CDR3 region (CDR = complementarity determining region) of the cAbLys3 camel antibody fragment, which binds to the active site of hen eggwhite lysozyme (HEL) and acts as a potent enzyme inhibitor by mimicking an oligosaccharide substrate, was prepared by solid-phase peptide synthesis. To obtain a closed loop-like structure resembling that in the crystal structure, N- and C-terminal cysteine residues were added to the linear peptide and oxidised to a cyclic disulfide-bridged peptide by using dimethylsulfoxide. A further, internal cysteine-12 residue was acetamidomethyl-protected to prevent possible oxidative byproducts. Affinity separation on a lysozyme microcolumn combined with MALDI-TOF mass spectrometry revealed that the peptide resumed high affinity to lysozyme only after deprotection of Cys-12, suggesting the importance of this paratope sequence for epitope recognition. The complex of lysozyme and active peptibody was characterised directly by conducting high-resolution ESI-FTICR mass spectrometry, which provided a molecular comparison of affinities for linear and cyclic peptibodies.

Keywords: antibodies · disulfide-bridge cyclization · lysozyme · mass spectrometry · peptides

Introduction

The general assumption that functional immunoglobulins always comprise two heavy and two light chains[1] was disproved by the discovery of the camel heavy-chain antibodies.[2] Antibodies from *Camalidae* that consist of heavy-chain homodimers are devoid of light chains and the C_H1 domains of mammalian immunoglobulins, and yet, they constitute up to 75% of the circulating immunoglobulins. Heavy-chain antibodies observed in other species, for example, in human serum, have no apparent function and have been considered as pathophysiologically aberrant forms.[3] Although the variable domain of a camel heavy-chain antibody is homologous in amino acid sequence and structure to the variable domains of heavy chains of human or mouse antibodies,[4] it contains several substitutions of polar amino acids in place of hydrophobic residues, which, in human antibodies, are shielded by the light-chain variable domain (VL).[5] Variable domains of camel heavy-chain antibodies have been termed V_HH to distinguish them from the classical VH domains; they provide a more hydrophilic surface leading to increased solubility and stability of the antibody structure.[4,5]

The lack of light chains means a reduction in the number of antigen binding sites on the heavy-chain antibodies, with only three (instead of six) complementarity determining regions (CDRs) available for antigen binding. The hypervariable loop-CDRs, especially CDR1 and CDR2, exhibit a restricted number of specific conformations (canonical structures) in mouse or human VHs, with different lengths and amino acid compositions.[6,7] In camel V_HHs, the structures of these loops differ significantly from those of mammalian VHs. In addition, the CDR3 loop of the V_HH domain is significantly larger than those in mouse and human VH, which increases the antigen-binding surface relative to that of VH and partially compensates for the lack of the light chain. A further difference between classical VH and camel V_HH is the frequent occurrence of an additional disulfide bridge be-

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tween CDR1 and CDR3, which probably restrains the flexibility of the CDR3 loop of a V_HH.

Several crystal structures of the V_HH have now been determined either alone or as antigen complexes.[8–10] A well-characterised case is the cAbLys3 camel antibody that binds to the active site of hen eggwhite lysozyme (HEL) and acts as a potent enzyme inhibitor by mimicking an oligosaccharide substrate.[11] A ribbon representation[12] of the crystal structure of the non-covalent complex formed by a single V_HH domain of cAbLys3 with HEL is shown in Figure 1a. This antibody binds lysozyme with high affinity ($K_D \approx 10^{-8}$ M) as shown by surface plasmon resonance (SPR) measurements of the non-covalent interaction between cAbLys3 and lysozyme.[13] The high binding specificity of lysozyme to the cAbLys3 was demonstrated by affinity mass spectrometry with immobilised lysozyme upon affinity binding of a mixture of the camel single-chain antibody and ubiquitin as a control protein. In contrast to the molecular ions of both proteins in the mixture (Figure 1b), the MALDI-TOF mass spectrum of the affinity elution fraction (Figure 1c) showed exclusively the molecular ion signals of the cAbLys3 antibody.

A characteristic feature of the single-domain antibody is that its CDR3 region forms an exposed loop of 24 amino acids that fits tightly into the active site cleft of lysozyme (Figure 1a). The cysteine-12 residue (Figure 2) divides this loop into two parts: an N-terminal half comprising around 70% of the surface in contact with the antigen; and a C-terminal half that shields a hydrophobic region of the antibody fragment (normally shielded by the VL in classical immunoglobulins) from solvent.[8] This characteristic structure prompted our interest to design and investigate the interaction of a single CDR3 loop with lysozyme.

Previous studies of lysozyme-binding camel heavy-chain antibodies revealed the existence of two different types of antibodies:[8] the first recognises a conformational epitope, encompassing a defined surface structure;[14] the second binds to a specific linear epitope sequence. The cAbLys3 antibody that inactivates the catalytic site of lysozyme has been shown to bind to a conformational epitope.[15] As reported previously, epitopes recognised by complete antibody structures may also be recognised by single CDR regions.[16] Such peptide antibodies, comprising around 20 amino acids, have been successfully designed and explored, for example, for HIV-protein antigens.[16,17] Cyclic peptide epitopes have been shown previously to exhibit high affinity, possibly by stabilising secondary structures essential for recognition.[18,19] We have designed a 26-amino acid residue peptide antibody loop ("peptibody"[1]) by addition of N- and C-terminal cysteine residues for cyclisation that has high, native-like affinity to the active site of HEL. By using a combination of affinity mass spectrometry methods for molecular comparison of linear and cyclic peptibodies we show that a specific paratope structure with suitable flexibility is essential for high affinity. The direct characterisation of the peptibody–HEL complex was obtained by high-resolution ESI-FTICR mass spectrometry, indicating this method to be an efficient tool for the study of corresponding biopolymer complexes.[20–23] Furthermore, by using a combination of proteolytic degradation of the immobilised microantibody–lysozyme complex

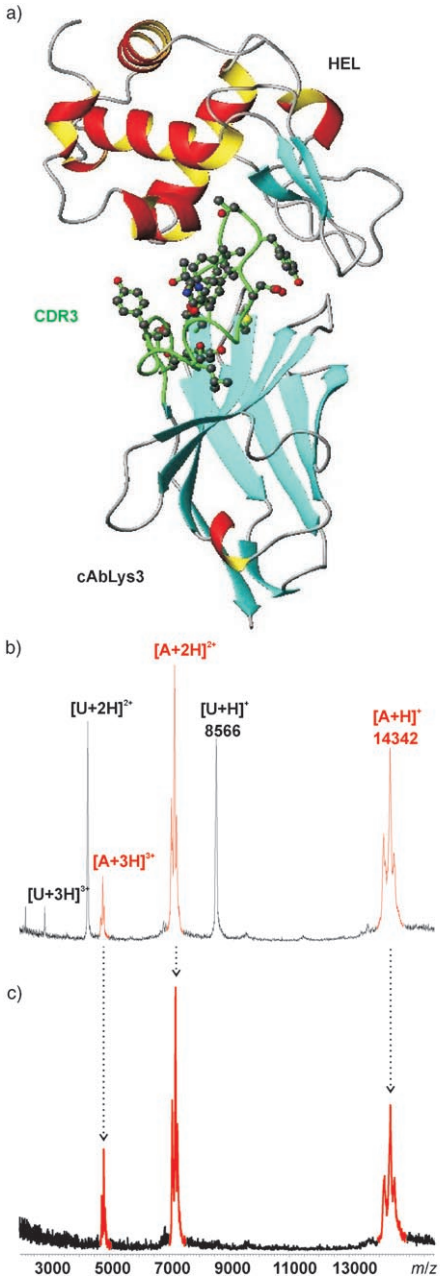

Figure 1. Structure and affinity mass-spectrometric characterisation of the camel-antibody complex with hen eggwhite lysozyme (HEL). a) Ribbon representation of the crystal structure of the non-covalent complex formed by cAbLys3 and HEL. The CDR3 region of the cAbLys3 camel antibody with side-chains is marked in green. b) MALDI-TOF mass spectrum of the protein mixture presented for affinity binding with immobilised HEL; A = cAbLys3, U = ubiquitin as a control. c) MALDI-TOF mass spectrum of the TFA elution fraction after affinity binding of the mixture.

[1] The term "peptibody" has been employed here to denote the synthetic functional peptides derived from the heavy-chain anti-HEL antibody.

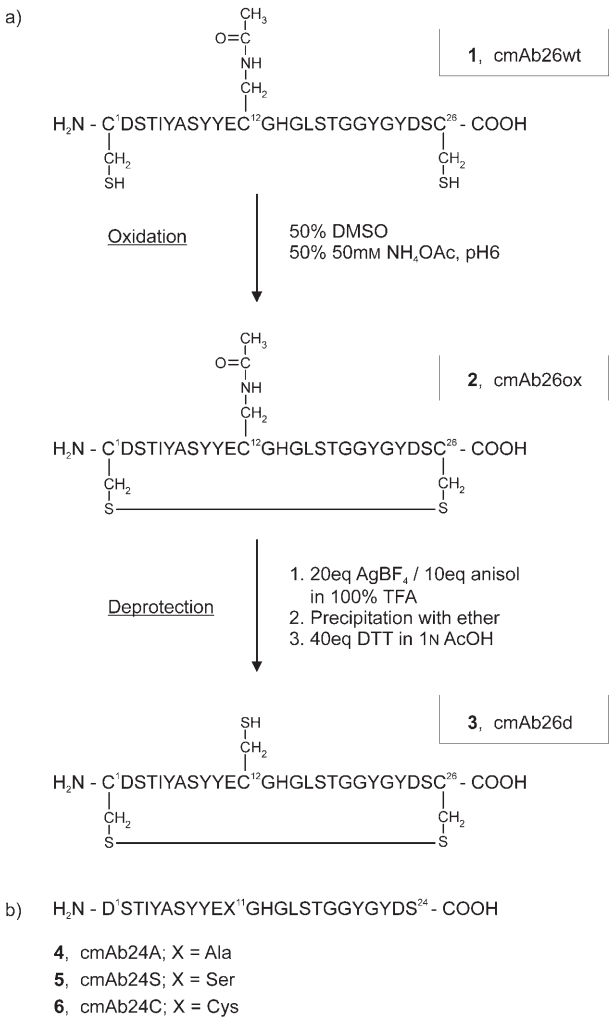

Figure 2. Procedure for the synthesis of peptibodies and subsequent DMSO oxidation (as described in the Experimental Section).

(analogous to epitope excision[24,25]) and mass-spectrometric peptide mapping, the essential residues of the paratope could be identified within a sequence of 10 amino acid residues in the N-terminal region of the peptibody.

Results and Discussion

Synthesis and characterisation of camel peptibody sequences: The single CDR3 loop C^{1}DSTIYASYYEC^{12}GHGLSTGGYGYDSC^{26} (Figure 2) of the camel heavy-chain antibody cAbLys3 was synthesised by solid-phase peptide synthesis (SPPS), and comprises two additional cysteine residues at the N- and C-termini of the peptide, respectively. 9-Fluorenylmethoxycarbonyl (Fmoc) and standard side-chain protection was generally employed for synthesis on a TGR resin; the internal Cys-12 residue was protected by acetamidomethyl (Acm), which is stable at the conditions used for cleavage of the peptide from the resin (90% trifluoroacetic acid (TFA), 5% water, 5% triethylsilane). The linear, Cys-12-Acm-protected peptide was subjected to cyclisation of the terminal Cys residues and subsequent deprotection of Cys-12 (Figure 2). In the first step, the Cys-12-protected peptide (cmAb26wt, **1**) was oxidised with DMSO to the Cys-1,26-disulfide-linked peptide (cmAb26ox, **2**), followed by deprotection of Cys-12 (cmAb26d, **3**), and both cyclic peptides were evaluated for their affinity to lysozyme.

The crude linear Cys-12-Acm peptide **1** was purified by HPLC on a C18 reversed-phase column. A single major product peak with a retention time of 23.9 min was isolated. Characterisation by MALDI-TOF-MS yielded an $[M+H]^+$ ion of the peptide with $m/z = 2844$, in agreement with the calculated molecular weight of 2843 Da, thus confirming the correct structure (Table 1). The spectrum also contained an additional ion at $m/z = 2865$ that corresponds to the $[M+Na]^+$ adduct that is frequently observed by MALDI-TOF-MS of polar peptides and proteins in the presence of traces of alkali salt.

The purified peptide was subjected to cyclisation by oxidation of the free terminal Cys residues. The crystal structure of the cAbLys3 antibody–lysozyme complex exhibited a cyclic CDR3 loop, which suggests that a cyclic peptibody has a higher affinity than the linear peptide.[10] Several approaches have been used for the oxidation of cysteine-thiol groups to the corresponding disulfides, for example, air oxidation, oxidation with reduced and oxidised glutathione[26] and with iodine.[27] However, significant problems in using these oxidising reagents are slow reaction kinetics and the formation of byproducts. With the present peptibody, DMSO was found to be an efficient and specific reagent without the above disadvantages,[28,29] providing mild oxidation conditions and fast reaction in a slightly acidic medium. DMSO oxidation was performed with the linear, Cys-12-acetamidomethylated peptide **1** under different conditions to evaluate the optimal conditions for cyclisation (data not shown). The yield was compared with that from air oxidation by performing alkylation of the unreacted cysteine-thiol groups with 4-vinylpyridine[28,29] followed by MALDI-TOF-

Table 1. Mass-spectrometric characterisation of camel-microantibody peptides, and ESI-FTICR-MS analysis of HEL–microantibody complexes.

No.	Peptide	Cys-12/Cys-11	MALDI-TOF-MS of microAb peptides		ESI-FTICR MS of HEL–microAb complexes	
			$[M+H]^+_{calcd}$	$[M+H]^+_{exptl}$	L[a]	C[b]
1	cmAb26wt	Acm	2843	2844	–	–
2	cmAb26ox	Acm	2842	2842	–	–
3	cmAb26d	–	2773	2771	1789.0992 (8+)	2135.3693 (8+)
4	cmAb24A	–	2535	2536	–	–
5	cmAb24S	–	2551	2552	–	–
6	cmAb24C	–	2567	2567	1788.3357 (8+)	2109.3526 (8+)

[a] Most abundant $[L+H]^{8+}$ ion of HEL. [b] Most abundant ion of HEL–microantibody-peptide complex.

MS analysis. These results indicated a significantly (ca. threefold) higher yield with DMSO oxidation than with air oxidation.

The HPLC-purified cyclic peptide **2** (cmAb26ox) was characterised by MALDI-TOF-MS (Table 1) and proteolytic degradation with chymotrypsin, which cleaves at tyrosine residues adjacent to the Cys-disulfide linkage and, therefore, was suitable to ascertain the cyclic structure of the peptide. The MALDI-TOF mass spectrum of the digest mixture (Figure 3a) showed three major fragments at: $m/z = 2376$ ([1–6]S–S[26–11]), $m/z = 2538$ ([1–6]S–S[26–10]) and $m/z = 2860$ ([1–9]S–S[26–10]), all of which contain the disulfide linkage

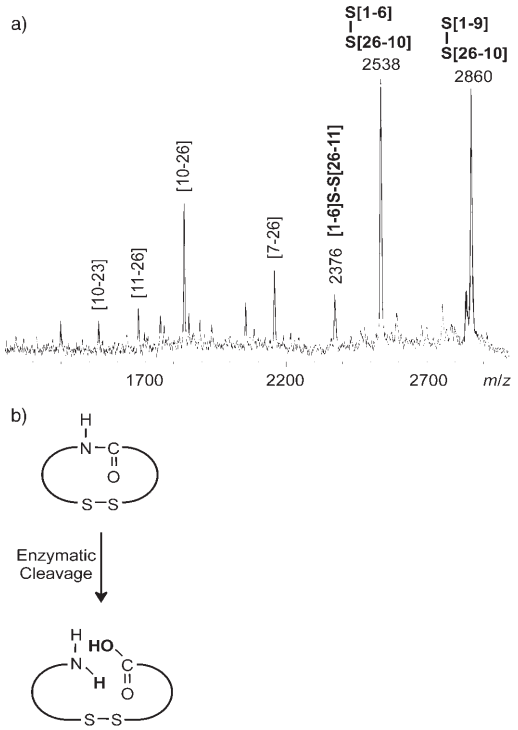

Figure 3. a) MALDI-TOF mass-spectrometric identification of the chymotryptic digestion products of the cmAb26ox peptibody **2**. b) Reaction scheme for the digestion of the cyclic peptide.

and confirmed the correct structure of the peptide. In particular, the ion at $m/z = 2860$ verified the cyclic peptide; a single cleavage yields a fragment with a mass of 18 amu greater than that of the peptide prior to cleavage ($m/z =$ 2844), due to addition of one H_2O molecule at the hydrolysis site (Figure 3b).

Characterisation of linear and cyclic peptibodies by affinity mass spectrometry: The cyclic peptide **2** (cmAb26ox) was analysed by affinity mass spectrometry to characterise its interaction with lysozyme. Lysozyme was immobilised on a Sepharose matrix in a short (0.8 mL) column, then subjected to affinity-binding experiments using MALDI-TOF-MS for analysis. Although peptide **2** contained the Cys-12-Acm protecting group as the only modification of the peptide sequence, no affinity to lysozyme was obtained. This lack of binding might be explained by the cyclisation of the peptide inhibiting the binding, or by the protecting group of the internal cysteine-12 residue causing the lack of affinity.

The Cys-12 residue was deprotected with tetrafluoroborate/TFA, yielding a MALDI-TOF mass spectrum of the crude product (Figure 4a) with a predominant $[M+H]^+$ ion of $m/z = 2773$, which corresponds well with the expected mass of the deprotected peptide **3**. Following deprotection, an affinity experiment yielded the MALDI-TOF mass spectra shown in Figure 4b–d. No peptide was found in the supernatant (washing) fraction (Figure 4c); the peptide **3** was identified exclusively in the elution fraction (Figure 4d), which confirmed the binding specificity and demonstrated that a slight modification of the paratope may completely abolish the binding affinity.[30,31] This peptide **3** can be regarded as a peptide antibody (peptibody), because it is derived from the native camel single-domain antibody, yet consists of only a short amino acid sequence.

Role of peptibody structure in the recognition of lysozyme: The comparable affinities shown by the deprotected cyclic and the linear peptide (Table 2) suggested similar structures for the linear and cyclic sequence. This assumption was supported by a molecular modelling study using the Hyperchem 7 programme for geometry optimisation (in vacuo), which indicated a loop-like structure for both peptides (Figure 5). Although the amino acid residues near the N- and C-termini showed differences in their spatial arrangement for the linear and the cyclic peptide, the structures of the internal "core" sequences were nearly identical. Consistent with the affinity studies, these results suggested that the amino acid sequence of the peptibody may be responsible for formation of a loop structure, and hence a cyclisation may not be essential for binding affinity.

The role of the internal cysteine residue in the binding affinity of the peptibody to lysozyme was further assessed by synthesis and affinity comparison of linear and cyclic peptides with and without a Cys-12 protecting group (Table 2). To estimate the binding affinities of the peptides, affinity mass spectrometry with immobilised HEL was performed as described above. The cyclic and linear peptibodies yielded dissociation constants in the micromolar range (Table 2), except for the cyclic Cys-12-protected peptide **2**, which revealed a drastically diminished (>1000-fold) affinity. Despite the decreased affinities of the peptibodies relative to the native camel single-chain antibody cAbLys3 ($K_D = 10^{-8}$ M), optimisation of affinities should be amenable by a number of chemical approaches, such as suitable amino acid mutations and structural changes in peptide sequences. Among the Cys-containing peptides, only the cyclic peptide containing the Cys-acetamidomethyl group showed loss of binding affinity. This result is in some contrast to the effect of the internal cysteine in the native camel antibody cAbLys3, modified by formation of a disulfide bridge, and may have two possible explanations:

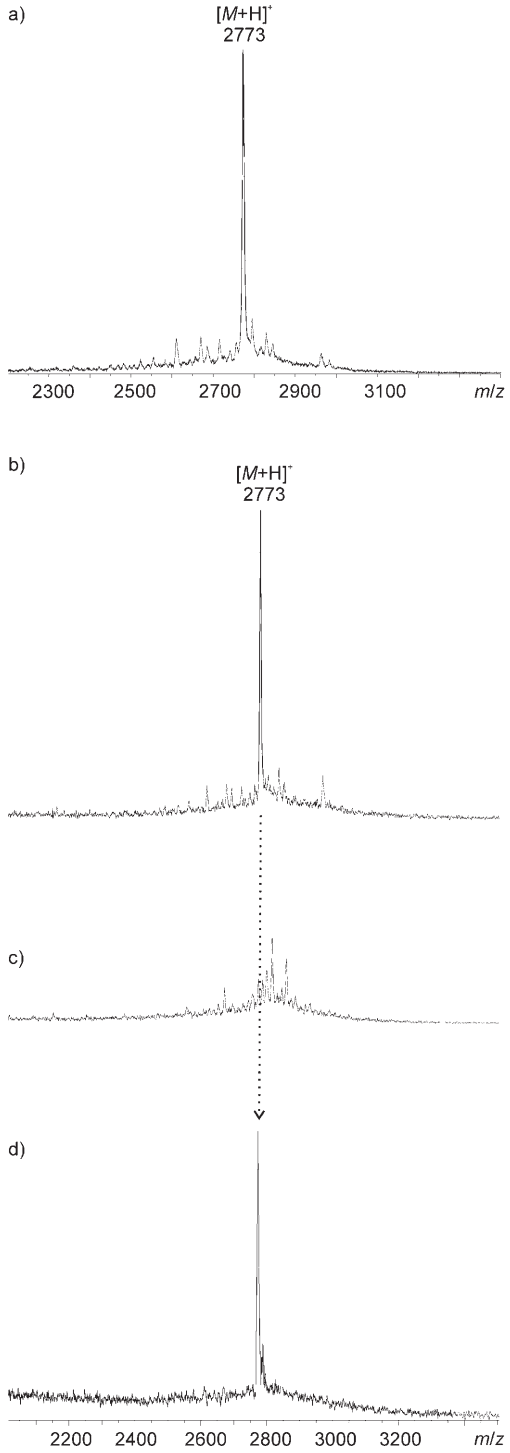

Figure 4. Affinity mass-spectrometric characterisation of the Cys-12-deprotected peptide cmAb26d **3**: a) MALDI-TOF mass spectrum of cmAb26d; b–d) Affinity MS analysis of **3** with immobilised HEL, showing the initial peptide substrate (b), supernatant after binding (c), TFA elution fraction (d).

The structure of the peptibody in the complex with lysozyme may be somewhat different from the structure of the peptide in the native cAbLys3 antibody–lysozyme complex, hence modification at specific amino acid residues may modify its affinity.

Table 2. Mass-spectrometric estimation of binding affinities of linear and cyclic camel-microantibody peptides.

No.	Peptide	Cys-12/Cys-11	Peptide structure	Affinity K_D [M][a]
1	cmAb26wt	Acm	linear	–
2	cmAb26ox	Acm	cyclic	$\gg 10^{-1}$ [b]
3	cmAb26d	–	cyclic[c]	$< 10^{-5}$
4	cmAb24A	–	linear	10^{-4}
5	cmAb24S	–	linear	10^{-5}
6	cmAb24C	–	linear	$< 10^{-5}$

[a] Affinity estimation from ion-intensity ratios of HEL–microantibody complexes. [b] No binding affinity. [c] Cyclic peptide containing around 20% linear sequence.

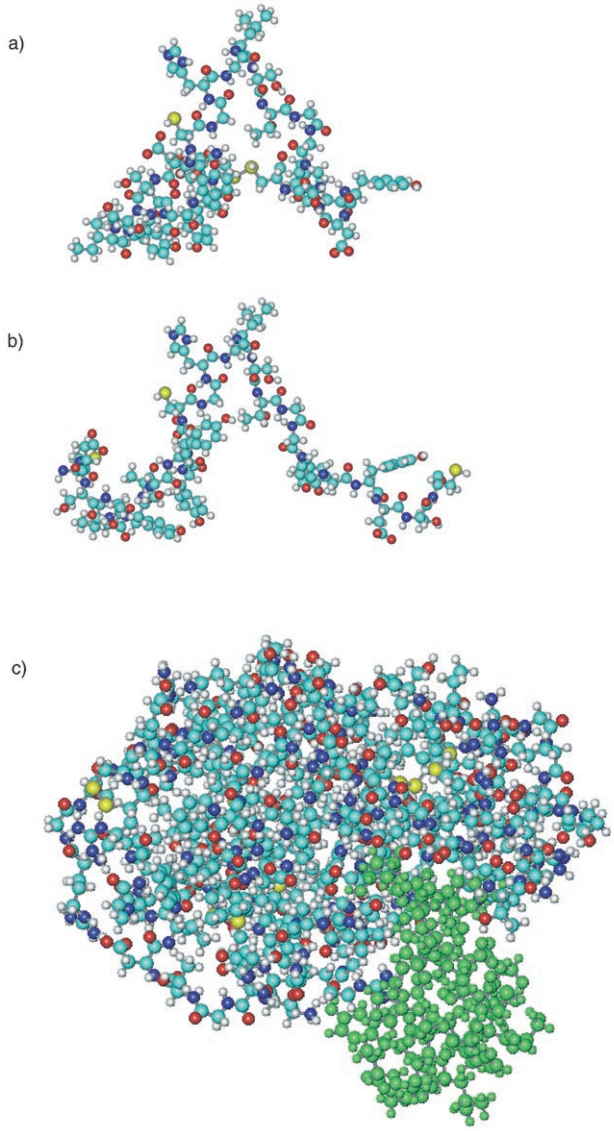

Figure 5. Molecular modelling calculations of conformations for the a) cyclic and b) linear peptibody cmAb26d, and c) for the structure of the HEL complex with the linear cmAb24d peptide, shown in green.

Covalent modification of single amino acid residues may cause a steric effect on antigen binding, which is compensated by reorganisation of the backbone structure in the linear

peptide; this reorganisation is thus inhibited by a reduced flexibility of the cyclic peptide.

Consistent with the latter effect, affinity studies with homologous synthetic peptides containing isosteric modifications of the cysteine-12 residue by Ala and Ser (**4**, **5**, see Figure 2) showed significant reductions in lysozyme-binding affinities (Table 2). These results, and the strong influence of the protecting group on binding affinity, clearly suggest a steric effect of a binding structure with reduced flexibility.

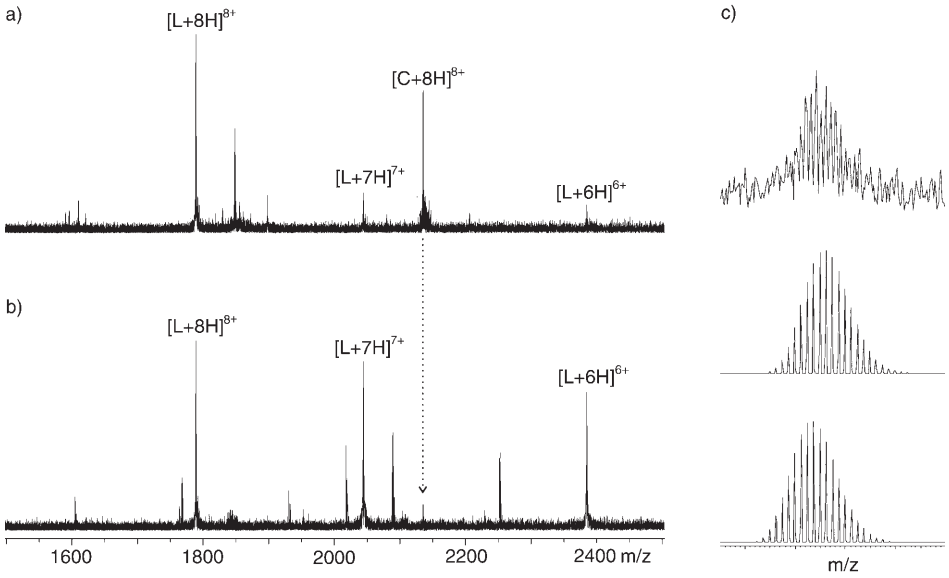

Figure 6. ESI-FTICR mass spectra of the HEL–peptibody cmAb26d complex (L = lysozyme, C = complex). a) Protonated molecular ions of the non-covalent complex at a desolvation potential of 120 V; b) FTICR mass spectrum at a desolvation potential of 210 V, in which disappearance of the complex-ion signals confirms the non-covalent character of the complex; c) comparison of experimental ion signals of the complex $[C+8H]^{8+}$ with calculated ion signals for the complex with a cyclic and linear peptibody at isotopic resolution.

Mass-spectrometric characterisation of the lysozyme–peptibody complex: To characterise directly the molecular binding composition and stoichiometry of the active microantibody, complexes of peptide with lysozyme were prepared and analysed by ESI-FTICR mass spectrometry.[23,32,33] Electrospray ionisation has been shown to be very suitable for the direct study of non-covalent interactions and supramolecular complexes, because this soft ionisation method preserves non-covalent complexes in their near-native structural states.[23,32,33] In addition, the application of high-resolution FTICR-MS provides complete isotopic resolution of all ions (with different charge states) of the intact complex[20,34] as well as components of the complex. The study of non-covalent complexes by ESI-MS requires complete dissolution of the molecules in a near-physiological solvent buffer. For the peptibody–lysozyme complexes, an ammonium acetate solution (10 mM, pH 6) was used, with the addition of a small amount of methanol as an organic spray-modifying solvent, to obtain suitably charged molecules for ion detection and a stable spray. In contrast, a large amount of organic cosolvent might destroy non-covalent interactions.[35,36]

FTICR mass spectra of the complex formed by the cyclic peptibody cmAb26d **3** and lysozyme are shown in Figure 6. The spectrum in Figure 6a was obtained at a desolvation potential of 120 V between the spray entrance nozzle and the skimmer electrode;[37] at these conditions the protonated molecular ions of the intact, stoichiometric complex of peptibody and lysozyme ($[C+8H]^{8+}$) could be identified clearly. The exact mass determinations of the complex ions by FTICR-MS are summarised in Table 1. The isotopically resolved spectrum (Figure 6c) showed two overlapping and abundant signals, corresponding to the complexes containing the reduced (linear) and oxidised (cyclic) peptibody, the latter resulting from partial reduction of the disulfide bridge during deprotection of the internal Cys-12 residue. In addition, the ion signals of free (excess) lysozyme were identified ($[L+xH]^{x+}$, $x = 6$–8). Increase in the desolvation potential to 210 V caused nearly complete dissociation of the complex (Figure 6b), as evidenced by the disappearance of the ion signal of the complex, which provides confirmation for the non-covalent nature of the complex.[23] The direct mass-spectrometric characterisation of the intact complexes of both the cyclic and linear microantibody thus provided evidence that a cyclic structure is not an essential prerequisite for obtaining high affinity to lysozyme.

Identification of the paratope-recognition structure of the anti-HEL peptibody: The surprising result that the peptibody may comprise a linear structure prompted our interest in characterising the paratope structure in more detail. For the molecular determination of epitopes, two similar procedures have been established previously in our laboratory, mass-spectrometric epitope excision and epitope extraction.[38–40] These methods have been successfully employed for identifying epitope sequences as well as conformational epitopes of antigens,[14,41,42] but have not yet been applied for the identification of paratope structures. In epitope excision, the antigen is bound to the immobilised antibody and the resulting immune complex subjected to proteolytic digestion. Shielding of specific cleavage sites in the antigen by the antibody enables the specific binding of the epitope to be maintained, which, following dissociation from the antibody, is identified by mass spectrometry. Epitope extraction involves a converse experimental procedure, in which the antigen is first proteolytically digested and a mixture of specific peptide fragments is presented to the antibody. The fragments containing the epitope are then bound by the an-

tibody and, after dissociation of the immune complex, identified by mass spectrometry. In the present lysozyme–peptibody complex, the corresponding methods, applied to the immobilised HEL antigen, have been termed paratope excision and paratope extraction, respectively.[42]

The original CDR3 region of the cAbLys3 antibody, DSTIYASYYECGHGLSTGGYGYDS (cmAb24C, **6**), was used as the peptibody. This corresponds to the linear cmAb26d peptide without the terminal cysteine residues and binds to lysozyme with high affinity (K_D of approximately 10^{-5} M as estimated by affinity mass spectrometry, see Table 2). Proteolytic digestions were performed with α-chymotrypsin and Glu-C protease (cleaving at C-terminal glutamyl–peptide bonds), and the fragment mixtures were submitted to the lysozyme column. Corresponding MALDI-TOF-MS results obtained with α-chymotrypsin are shown in Figure 7. The spectrum of the paratope elution fraction (spectrum c) showed only a single fragment, [1–20], with substantial affinity to lysozyme. No peptide fragments corresponding to proteolytic cleavage within the N-terminal region of the peptide were found in the elution fraction. By Glu-C digestion, only the intact peptide, but neither of the two expected proteolytic fragments [1–10] and [11–20], was identified, indicating that the shielded Glu-10 residue is part of the paratope. In summary, the proteolytic and mass-spectrometric data obtained by paratope extraction show that the N-terminal residues Y-5, Y-8, Y-9, E-10 are all shielded by HEL binding, but are cleaved in control experiments in which HEL is absent. By contrast, the corresponding C-terminal residues are cleaved in both cases. These results provide evidence that the paratope of the cmAb24C peptibody is contained in the N-terminal region and located in the partial sequence DSTIYASYYEC.

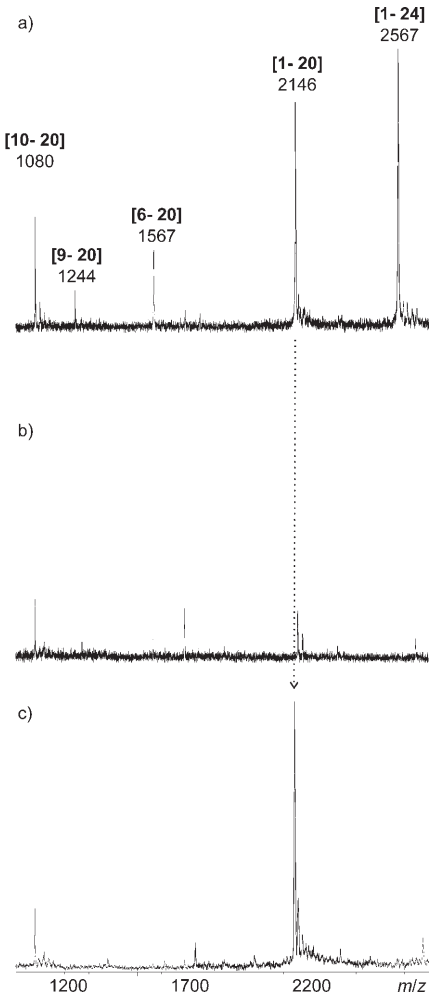

Figure 7. Paratope extraction-MS analysis of cmAb24C with immobilised HEL following chymotrypsin digestion. a) Chymotryptic digest mixture of the microantibody peptide; b) supernatant fraction; c) TFA elution fraction after affinity binding of the digest mixture in a).

Conclusions

We provide evidence that a single synthetic peptide can essentially maintain the antigen recognition of a complete camel heavy-chain antibody, despite comprising a relatively short sequence. This peptibody showed high affinity to the antigen. The recent development of high-resolution mass spectrometry applied to both paratope excision and paratope extraction with proteolytically stable, immobilised antigens provides powerful new approaches to directly identify antibody-recognition structures.[43] The application of paratope extraction together with mass spectrometry in this study revealed that the paratope of the microantibody is located within an 11-amino acid sequence in the N-terminal region.

The structure of the peptibody peptide appears to be essential for its binding properties. Although a cyclic structure was identified by X-ray crystallographic analysis of the antibody–antigen complex, the present results of affinity mass spectrometry and structure modelling show that the linear peptibody is well suited to forming a high-affinity complex with lysozyme. This peptide may form a loop-like structure, provided sufficient flexibility can be maintained. Thus, although differences in the relative binding strengths may exist, flexibility appears to be an important prerequisite for the binding structure of the camel heavy-chain anti-HEL antibody.

Experimental Section

Peptide synthesis: Antibody peptides with N- and C-terminal cysteine residues (cmAb26wt, **1**) C^{1}DSTIYASYYE$\mathbf{C^{12}}$(acetamidomethyl)-GHGLSTGGYGYDSC^{26}, and the original camel CDR3-sequence of cAbLys3 peptide (cmAb24C) DSTIYASYYECGHGLSTGGYGYDS, were synthesised as carboxamides by using a semiautomatic peptide synthesiser (EPS 221; Intavis, Langenfeld, Germany) according to the Fmoc-synthesis strategy.[44,45] Synthesis was carried out on a NovaSyn TGR resin, with benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium-hexafluorophosphate (PyBOP) and N-methyl-morpholine used as coupling reagents. All coupling steps were carried out in DMF. N-α-Fmoc-protected amino acids were used with the following side-chain protections: Asp(O*t*Bu), Thr(*t*Bu), Ser(*t*Bu), Glu(O*t*Bu), Cys(Trt), Cys(Acm), Tyr(*t*Bu) and His(Trt). Deprotection of Fmoc was generally carried out with 20%

piperidine in DMF, and cleavage of peptide from the resin and simultaneous side-chain deprotection (with the exception of Cys-acetamidomethyl) was performed with 90% TFA, 5% triethylsilane and 5% water. Molecular homogeneities of all purified peptides were ascertained by MALDI-TOF-MS.

Oxidation of terminal cysteinethiol groups: The crude peptide (cmAb26wt; 2 mg, 0.7 μmol) was dissolved in a mixture of 0.5 mL NH_4OAc (50 mM, pH 6) and 0.5 mL DMSO, and the reaction mixture was shaken overnight at 20°C and lyophilised. The residue was redissolved in 0.1% aqueous TFA and purified by RP-HPLC. As a control, 10 μg of the oxidised peptide cmAb26ox (**3**) was alkylated with 1 μl 4-vinylpyridine in 50 μl NH_4HCO_3 (50 mM, pH 8) for 1 hr at 20°C, and the product lyophilised, redissolved in 0.1% TFA and characterised by MALDI-TOF-MS.

Cysteine deprotection: Removal of the internal Cys-12-acetamidomethyl protecting group was achieved by using silver tetrafluoroborate. Crude cmAb26ox peptide (1 mg, 0.35 μmol) was dissolved in 100 μl ice-cold TFA, silver tetrafluoroborate (7 μmol) and anisole (3.5 μmol) were added and the reaction mixture was shaken for 1 hr at 0°C. The peptide silver salt was then precipitated by using *tert*-butyl methyl ether, isolated by centrifugation and treated with dithiothreitol (14 μmol) in 1 M acetic acid for 3 hr at 20°C. After centrifugation, the supernatant containing the deprotected peptide (cmAb26d) was desalted and purified by RP-HPLC.

HPLC purification: HPLC was performed by using a Waters-Millipore M590/510 instrument equipped with an M-490 UV detector. Peptides were separated on a 25×0.4 cm Vydac RP-C18 nucleosil column (Macherey-Nagel, Duisburg, Germany) by using a linear binary gradient of 0.1% aqueous TFA (A) and 0.1% TFA in acetonitrile (B), 20–40% B/30 min at a flow rate of 1 mL min^{-1}. Peptides were detected at 220 nm, and fractions were lyophilised for MALDI-TOF-MS and FTICR-MS analysis.

Affinity studies: Approximately 500 μg (0.034 μmol) of lyophilised hen eggwhite lysozyme (Sigma, St Louis, USA) was dissolved in 0.3 mL coupling buffer (0.2 M $NaHCO_3$, 0.5 M NaCl, pH 8.3), the solution was added to 120 mg dry NHS-activated 6-aminohexanoic acid-coupled Sepharose (Sigma, Germany) and the coupling reaction was performed for 60 min at 25°C. The Sepharose-coupling product was loaded into a microcolumn (Mobitec, Göttingen, Germany) and the matrix was washed consecutively with 10 mL each of washing buffer (0.5 M aminoethanol, 0.5 M NaCl, pH 8.3) and blocking buffer (0.1 M CH_3COONa, 0.5 M NaCl, pH 4.0). The microcolumn was stored at 4°C in PBS buffer (1 mM Na_2HPO_4, 136 mM NaCl, 2.7 mM KCl, 0.01% NaN_3, pH 7.3) until use.

For affinity experiments, 20 μg of peptide was dissolved in 50 μl PBS buffer (1 mM Na_2HPO_4, 136 mM NaCl, 2.7 mM KCl, pH 7.3) and loaded onto the affinity column. The column was shaken for 2 hr and then washed with 20 mL PBS buffer to remove unbound peptide. Affinity-bound peptide was eluted with 10 mL 0.1% aqueous TFA, the elution fraction was isolated and the column was reconditioned with at least 20 mL PBS buffer. The first few millilitres of the wash and elution fractions were lyophilised and characterised by MALDI-TOF-MS.

Proteolytic digestion: A 1/10 to 1/5 aliquot of 100 μg (0.034 μmol) HPLC-purified peptide (cmAb26ox) was dissolved in 100 μl $NaHCO_3$ buffer (50 mM, pH 8), and chymotrypsin was added at a substrate-to-enzyme ratio of 1:100. The reaction was performed for 15 min at 37°C. After lyophilisation, the residue was redissolved in 50 μl 0.1% aqueous TFA and characterised by MALDI-TOF-MS.

Paratope excision and extraction: All enzymatic digestion reactions for the determination of paratope peptide fragments were carried out at 37°C with 20 μg (0.007 μmol) of peptibody, using an enzyme-to-substrate ratio of 1:50. For the paratope-excision experiments, the peptibody was allowed to bind to the immobilised lysozyme for at least 2 hr, and unbound material was removed by extensive washing of the affinity column with PBS buffer. After addition of protease, the immobilised HEL–peptibody complex was digested for 2 hr at 37°C. Non-binding peptides were removed with 10 mL PBS buffer and paratope peptides were then eluted with 10 mL 0.1% TFA. The paratope-extraction experiments were performed in the same manner, except that digestion of the peptibody was carried out first, followed by addition of the peptide fragment mixture to the affinity column. The washing and elution fractions of all paratope-determination studies were lyophilised and the residue were dissolved in 0.1% TFA for mass-spectrometric analysis.

Mass spectrometry: For MALDI-TOF-MS analysis, the samples were dissolved in 0.1% aqueous TFA. MALDI-TOF-MS was performed with a Bruker Biflex TOF mass spectrometer (Bruker Daltonik, Bremen, Germany) equipped with a 26-sample SCOUT source and video system, a nitrogen UV laser ($\lambda_{max}=337$ nm) and a dual-channel plate detector. Sample solution (1 μl) was placed on the target and 1 μl of a freshly prepared saturated solution of 4-hydroxy-α-cyanocinnamic acid (HCCA) in acetonitrile/H_2O (2:1) with 0.1% TFA was added. Because of the high salt content in the analyte solution, sodium and potassium salts were removed without significant loss of peptide by using ZipTipC18 pipette tips (Millipore). The spot was then crystallised on-target by addition of 1 μL acetonitrile/H_2O (4:1), which resulted in a fine crystalline matrix. MALDI-TOF mass spectra were recorded at an acceleration voltage of 20 kV and a detector voltage of 1.5 kV by accumulation of 50–200 single laser shots into a resultant spectrum. External calibration was carried out by using the singly protonated-ion signals of bovine insulin (5734.6 Da), neurotensin (1672.9 Da), substance P (1617.9 Da), bradykinin (1240.4 Da) and angiotensin II (1046.2 Da).

ESI-FTICR mass spectra were recorded by using a Bruker Daltonik APEX II instrument (Bremen, Germany) equipped with a 7 Tesla actively shielded super-conducting magnet (Magnex, Oxford, UK). Details of the instrumental conditions for ESI-FTICR-MS were as reported previously,[34,46–48] for a Bruker Apollo ESI source with an API100 controller and a UNIX-based Silicon Graphics OS2 workstation data system. The spectra were acquired by using the Bruker Daltonik software XMass and corresponding programmes for mass calculation, data calibration and processing. Samples were dissolved in a solution of 70% NH_4OAc (10 mM, pH 6) with 30% methanol and analysed at a flow rate of 2 μL min^{-1}. For all measurements of non-covalent complexes, the concentration of lysozyme was 7 μM with a five molar excess of the peptibody.

Molecular modelling: Molecular modelling was calculated by using the software Hyperchem 7. A geometry optimisation was performed by using the Amber99 parameter set for the force field and the Polak-Ribiere algorithm with a RMS gradient of 0.05 kcal.

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[1] E. A. Padlan, *Mol. Immunol.* **1994**, *31*, 169–217.
[2] C. Hamers-Casterman, T. Atarhouch, S. Muyldermans, G. Robinson, C. Hamers, E. B. Songa, N. Bendahman, R. Hamers, *Nature* **1993**, *363*, 446–448.
[3] M. Seligmann, *Rev. Prat.* **1979**, *29*, 373–376, 381–382.
[4] S. Muyldermans, T. Atarhouch, J. Saldanha, J. A. Barbosa, R. Hamers, *Protein Eng.* **1994**, *7*, 1129–1135.
[5] V. K. Nguyen, S. Muyldermans, R. Hamers, *J. Mol. Biol.* **1998**, *275*, 413–418.
[6] C. Chothia, A. M. Lesk, *J. Mol. Biol.* **1987**, *196*, 901–917.
[7] C. Chothia, A. M. Lesk, A. Tramontano, M. Levitt, S. J. Smith-Gill, G. Air, S. Sheriff, E. A. Padlan, D. Davies, W. R. Tulip, *Nature* **1989**, *342*, 877–883.
[8] K. Decanniere, T. R. Transue, A. Desmyter, D. Maes, S. Muyldermans, L. Wyns, *J. Mol. Biol.* **2001**, *313*, 473–478.
[9] A. Desmyter, K. Decanniere, S. Muyldermans, L. Wyns, *J. Biol. Chem.* **2001**, *276*, 26285–26290.

[10] A. Desmyter, T. R. Transue, M. A. Ghahroudi, M. H. Thi, F. Poortmans, R. Hamers, S. Muyldermans, L. Wyns, *Nat. Struct. Biol.* **1996**, *3*, 803–811.
[11] T. R. Transue, E. De Genst, M. A. Ghahroudi, L. Wyns, S. Muyldermans, *Proteins* **1998**, *32*, 515–522.
[12] R. Koradi, M. Billeter, K. Wuthrich, *J. Mol. Graphics* **1996**, *14*, 29–32, 51–55.
[13] E. De Genst, D. Areskoug, K. Decanniere, S. Muyldermans, K. Andersson, *J. Biol. Chem.* **2002**, *277*, 29897–29907.
[14] W. Fiedler, C. Borchers, M. Macht, S. O. Deininger, M. Przybylski, *Bioconjugate Chem.* **1998**, *9*, 236–241.
[15] M. Z. Atassi, *Immunochemistry* **1978**, *15*, 909–936.
[16] M. Levi, M. Sallberg, U. Ruden, D. Herlyn, H. Maruyama, H. Wigzell, J. Marks, B. Wahren, *Proc. Natl. Acad. Sci. USA* **1993**, *90*, 4374–4378.
[17] N. A. Jackson, M. Levi, B. Wahren, N. J. Dimmock, *J. Gen. Virol.* **1999**, *80*, 225–236.
[18] M. L. Mangoni, N. Papo, G. Mignogna, D. Andreu, Y. Shai, D. Barra, M. Simmaco, *Biochemistry* **2003**, *42*, 14023–14035.
[19] M. M. Rosenblatt, J. Wang, K. S. Suslick, *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 13140–13145.
[20] J. E. Bruce, G. A. Anderson, R. Chen, X. Cheng, D. C. Gale, S. A. Hofstadler, B. L. Schwartz, R. D. Smith, *Rapid Commun. Mass Spectrom.* **1995**, *9*, 644–650.
[21] D. Lafitte, A. J. Heck, T. J. Hill, K. Jumel, S. E. Harding, P. J. Derrick, *Eur. J. Biochem.* **1999**, *261*, 337–344.
[22] L. Pasa-Tolic, A. C. Harms, G. A. Anderson, R. D. Smith, A. Willie, M. S. Jorns, *J. Am. Soc. Mass Spectrom.* **1998**, *9*, 510–515.
[23] M. Przybylski, M. O. Glocker, *Angew. Chem.* **1996**, *108*, 879–899; *Angew. Chem. Int. Ed. Engl.* **1996**, *35*, 806–826.
[24] D. Suckau, M. Mak, M. Przybylski, *Proc. Natl. Acad. Sci. USA* **1992**, *89*, 5630–5634.
[25] D. Suckau, J. Kohl, G. Karwath, K. Schneider, M. Casaretto, D. Bitter-Suermann, M. Przybylski, *Proc. Natl. Acad. Sci. USA* **1990**, *87*, 9848–9852.
[26] M. Ferrer, C. Woodward, G. Barany, *Int. J. Pept. Protein Res.* **1992**, *40*, 194–207.
[27] M. Pohl, D. Ambrosius, J. Grotzinger, T. Kretzschmar, D. Saunders, A. Wollmer, D. Brandenburg, D. Bitter-Suermann, H. Hocker, *Int. J. Pept. Protein Res.* **1993**, *41*, 362–375.
[28] A. Otaka, T. Koide, A. Shide, N. Fujii, *Tetrahedron Lett.* **1991**, *32*, 1223–1226.
[29] J. P. Tam, C. R. Wu, W. Liu, J. W. Zhang, *J. Am. Chem. Soc.* **1991**, *113*, 6657–6662.
[30] W. J. Syu, L. Kahan, *J. Immunol. Methods* **1989**, *118*, 153–160.
[31] J. K. Larson, L. Otvos, Jr., H. C. Ertl, *J. Virol.* **1992**, *66*, 3996–4002.
[32] J. E. Bruce, V. F. Smith, C. Liu, L. L. Randall, R. D. Smith, *Protein Sci.* **1998**, *7*, 1180–1185.
[33] M. Przybylski, V. Schnaible, J. Kast, S. Bühler, J. Michels, A. Wattenberg, T. A. Fligge, D. Forst, K. Diederichs, C. Zeth, M. O. Glocker, W. Welte, *Approaches to the Characterization of Tertiary and Supramolecular Protein Structures by Combination of Protein Chemistry and Mass Spectrometry* (Eds.: W. Ens, K. G. Standing, I. V. Chernushevich), Kluwer Academic, **1998**, pp. 17–43.
[34] M. K. Green, M. M. Vestling, M. V. Johnston, B. S. Larsen, *Anal. Biochem.* **1998**, *260*, 204–211.
[35] S. Martinovic, S. J. Berger, L. Pasa-Tolic, R. D. Smith, *Anal. Chem.* **2000**, *72*, 5356–5360.
[36] X. J. Tang, C. F. Brewer, S. Saha, I. Chernushevich, W. Ens, K. G. Standing, *Rapid Commun. Mass Spectrom.* **1994**, *8*, 750–754.
[37] J. B. Fenn, M. Mann, C. K. Meng, S. F. Wong, C. M. Whitehouse, *Science* **1989**, *246*, 64–71.
[38] C. E. Parker, K. B. Tomer, *Methods Mol. Biol.* **2000**, *146*, 185–201.
[39] M. Macht, W. Fiedler, K. Kurzinger, M. Przybylski, *Biochemistry* **1996**, *35*, 15633–15639.
[40] M. Macht, A. Marquardt, S. O. Deininger, E. Damoc, M. Kohlmann, M. Przybylski, *Anal. Bioanal. Chem.* **2003**, *378*, 1102–1111.
[41] J. McLaurin, R. Cecal, M. E. Kierstead, X. Tian, A. L. Phinney, M. Manea, J. E. French, M. H. Lambermon, A. A. Darabie, M. E. Brown, C. Janus, M. A. Chishti, P. Horne, D. Westaway, P. E. Fraser, H. T. Mount, M. Przybylski, P. St George-Hyslop, *Nat. Med.* **2002**, *8*, 1263–1269.
[42] J. Van de Water, S. O. Deininger, M. Macht, M. Przybylski, M. E. Gershwin, *Clin. Immunol. Immunopathol.* **1997**, *85*, 229–235.
[43] M. Przybylski, E. Amstalden, A. Marquardt, X. Tian, R. Stefanescu, M. Manea, R. Iacob, E. Damoc, unpublished results.
[44] G. B. Fields, R. L. Noble, *Int. J. Pept. Protein Res.* **1990**, *35*, 161–214.
[45] R. B. Merrifield, *Adv. Enzymol. Relat. Areas Mol. Biol.* **1969**, *32*, 221–296.
[46] A. G. Marshall, C. L. Hendrickson, G. S. Jackson, *Mass Spectrom. Rev.* **1998**, *17*, 1–35.
[47] C. L. Hendrickson, M. R. Emmett, *Annu. Rev. Phys. Chem.* **1999**, *50*, 517–536.
[48] E. Damoc, N. Youhnovski, D. Crettaz, J. D. Tissot, M. Przybylski, *Proteomics* **2003**, *3*, 1425–1433.

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